SCREENING FOR INHIBITORS OF TERPENOIDS BIOSYNTHESIS

BACKGROUND OF THE INVENTION

The present invention relates generally to the field of herbicides and specifically to a method of inhibiting the growth of plants and a method for rapid screening chemical libraries for inhibitors.

In the past herbicidal research has been carried out by first testing chemical compounds directly for their ability to inhibit the growth of plants and only much later was the biochemical mode of action of any herbicide, found in such manner, elucidated. With such a blind process the results are highly fortuitous and largely uncontrolled.

We have now succeeded to reverse this process to develop novel herbicides, with a predetermined mode of action, by a rational method of determination of herbicidal activity. Specifically our methodology proceeds in the following manner. We first determine general properties which an ideal herbicide should have. These can be spelled out as follows:

(1) The herbicide should be highly effective against all kinds of weeds.

(2) It should be non-toxic for humans and all animals that are potentially exposed to the herbicide.

(3) It should have a mode of action, which offers itself to the production of genetically engineered crop plants with a resistance to the herbicide.

We have succeeded in solving this problem. As a first aspect of our solution of this problem we determined a biochemical pathway that is an ideal target for a herbicide: the biosynthetic pathway to terpenoids via 1-deoxy-D-xylulose 5-

phosphate. It is not found in animals of any kind. It is of critical significance for all plants and it involves highly specialized and idiosyncratic enzymatic steps expected to be catalysed by idiosyncratic enzymes so that cross-inhibitions with other enzymes notably mammalian enzymes do not have to be feared.

In order to more fully appreciate this aspect of our solution of the above problem we will now consider the biochemical background of this pathway.

Classical studies by Bloch, Cornforth, Lynen and their coworkers have established that isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAP) are key intermediates in a biosynthetic pathway to terpenoids. More recently, an alternative pathway was shown to be operative in certain bacteria (Rohmer et al, 1993; Rohmer et al, 1996; Duvold et al, 1997; Sprenger et al, 1997) and in plants (Arigoni et al, 1997).

This novel pathway is shown in Figure 1. It begins with a condensation of pyruvate with glyceraldehyde 3-phosphate generating 1-deoxy-D-xylulose 5-phosphate (DXP), which seems to be subsequently converted into 2C-methyl-D-erythritol 4-phosphate (MEP) in a two-step reaction involving an intramolecular rearrangement and a reduction. Presumably, this intermediate is subsequently converted into IPP and DMAP. The classical pathway generates IPP and DMAP via mevalonate. It has been found that the alternative pathway does not occur in animals and that in plants the classical pathway occurs in the cytosol while the alternative pathway occurs in plastids (e.g. chromoplasts or chloroplasts).

SUMMARY OF THE INVENTION

This invention relates specifically to novel methods for screening and identifying compounds that specifically inhibit a metabolic target site or pathway in plants, notably an enzyme in the alternative bioysynthetic pathway to terpenoids via 1-deoxy-D-xylulose 5-phosphate.

Therefore, it is one of the main objects of the invention to provide a screening assay for identifying inhibitory compounds of the plant biosynthesis of terpenoids via 1-deoxy-D-xylulose 5-phosphate.

Accordingly, the present invention comprises a method for screening for the presence of inhibition of at least one enzyme in the biosynthetic pathway to terpenoids via 1-deoxy-D-xylulose 5-phosphate in plants comprising the following steps:

(a) preparing a suspension of cells or plastids of a plastid-bearing organism in a culture medium for supporting the metabolism of said cells or plastids at least to the extent of said biosynthetic pathway,

(b1) adding to said suspension a predetermined amount of a carbon-13-, carbon-14-, deuterium-, or tritium-labelled biochemical precursor for generating terpenoids via said pathway,

(c1) incubating the mixture obtained in step (b1) for a predetermined period of time at a predetermined temperature and

(d1) separating from said incubated mixture obtained in step (c1) a fraction comprising a product or intermediate downstream from 1-deoxy-D-xylulose 5-phosphate in said pathway,

(e1) detecting the concentration of labelled product(s) in said fraction obtained in step (d1),

(b2) repeating step (b1) with the addition of a predetermined amount of a chemical test sample under otherwise identical conditions,

(c2) to (e2) repeating steps (c1) to (e1) with the mixture obtained in step (b2) under the same conditions as in steps (c1) to (e1) and

(f) determining the presence of inhibition of at least one enzyme in said pathway by observation of whether the concentration of labelled product(s) detected in step (e1) is higher than that detected in step (e2).

It is a more specific object of the invention to provide a screening method that is highly sensitive to give significant results even with low conversion rates and/or low inhibitions. As a solution, we have found that the assay may be highly improved by adding a combination of ATP and CTP or a source for CTP. A lesser improvement is achieved by adding ATP alone or CTP or a source for CTP alone.

It is a further more specific object of the invention to provide a rapid screening assay.

We have now found that this problem can be solved by separating as the fraction comprising a product downstream simply water comprising tritiated water.

Further, the invention relates specifically to novel compounds which inhibit an enzyme in the alternative biosynthesis of terpenoids downstream from 1-deoxy-D-xylulose 5-phosphate.

Another aspect of the invention is a method for the control of undesirable plant growth which comprises applying to a locus where control is desired a herbicidally effective amount of an inhibitory compound identified by the screening method of the invention.

According to still another aspect of the invention, a herbicidal composition is provided comprising an inhibitor compound of the invention as an active ingredient in combination with an agriculturally acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts the first two steps of the alternative terpenoid pathway previously known.

Fig. 2 depicts a chemical synthesis of 2C-Methyl-D-erythritol 4-phosphate.

Fig. 3 depicts a generalized biosynthetic reacton sequence for the production of tritiated water.

Fig.4 illustrates a laboratory setup for performing the screening method of the invention.

Fig. 5 illustrates a high-throughput method with multi-well plates.

DETAILED DESCRIPTION OF THE INVENTION

This screening method may either be carried out with whole cells of a plastid-bearing organism or with separated plastids. In either case, the incubation with a labelled specific precursor in the alternative pathway via 1-deoxy-D-xylulose 5-phosphate means that only enzymes in this pathway are involved in the screening. The screening may be done with cell cultures or with a suspension of plastids. A cell culture of *Catharanthus roseus* may for example be used. The plastids may be chromoplasts, chloroplasts, etioplasts or leucoplasts. As substitutes cyanobacteria may be used in view of their phylogenetic relation to plastids. Preferred are plastids of weeds or algae or of plants closely related thereto.

Plastids or cells harboring plastids may be obtained from any organism having

plastids. Chromplasts are preferred due to a high yield of isoprenoids. Most convenient are chromoplasts from red peppers or similar fruits.

The plastids may be obtained from plant tissue by disruption of the cells, filtration and centrifugation according to known methods e.g. Camara, Method, Enzymol; 214, 352-365 (1993) or Liedvogel, Cytobiology 12, 155-174 (1976). These publications also disclose incubation media for these plastids.

The incubation may be carried out in any incubation medium suitable for incubating plastid or cell suspensions. It is merely required that the metabolism is operative at least to the extent of the alternative biosynthetic pathway. The incubation medium should support the metabolism. In the simplest and most preferred case the incubation medium should not inhibit the reactions involved and all cofactors would be provided by the plastids or cells. It is also possible to add one or several cofactors to the incubation medium. The pH of the incubation medium should be physiologically suitable, preferably in the range of 5 to 9 and especially in the range of 7 to 8.

Since the plastids are biochemically fully competent they will provide any additional components for the biosynthesis. It is preferred but not mandatory to add a magnesium salt and/or a divalent manganese salt for bringing the enzymes to optimum activity and it is further preferred to add NADPH and/or NADP each in concentrations of 0.5 to 4 mM preferably 1 to 2 mM. Further FAD may be added in a concentration of preferably 1 to 100 μM, especially 5 to 50 μM. It is advantageous to add NaF in order to block phosphatases. The concentration of NaF is preferably 1 to 20 mM.

Plastids or plastid bearing cells as such are biochemically fully competent also in terms of any nucleotides required by the biosynthetic reaction. It is preferred to

add ATP. It is also preferred to add CTP or a source for CTP. It is more preferrred to add ATP as well as CTP or sources thereof. In such embodiments the concentration of ATP or its source in the incubation medium is preferably 1 to 20 mM and especially 3 to 10 mM and the concentration of CTP or its source is preferably 1 to 20 mM and especially 3 to 10 mM. CMP may be used as a source for CTP. It is converted to CTP by the actions of (preferably externally added) ATP and the enzymes within the plastids. Similarly, CDP may be used as a source for CTP.

We have found a synergism of ATP and CTP (or its source) in terms of promoting the enzymatic reaction. This expands the scope of applicability of the screening method to reaction conditions or incubation times giving too low rates of the reaction without the combination of promotors; or to inhibitors giving only inconclusive or marginally conclusive results without the combination of promotors. This is very important, since it is more likely that in a screening of a chemical library any inhibitor first found is only marginally effective rather than highly effective, so that more effective inhibitors can be subsequently detected in the class of chemical variants of the first found inhibitor.

The plastids or cells isolated by methods known per se may be completely uninjured or more or less injured, even to the extent of being at least partially disrupted. The biochemical competence and the success of the screening procedure is largely independent of such differences and quite robust.

Any precursor of IPP in the alternative pathway can be used or any compound that is biochemically converted into such a precursor. It is merely required that it is appropriately labelled so that its conversion can be detected by detecting the label in a product of the biosynthetic pathway.

The labelled substrates may be prepared enzymatically or chemically.

Enzymatically, tritiated 1-deoxy-D-xylulose 5-phosphate may be prepared from [3-^{3}H]pyruvate for tritiation in position 1; or from [1-^{3}H]glyceraldehyde 3-phosphate or [1-^{3}H]dihydroxyacetone 3-phosphate for tritiation in position 3 or from [2-^{3}H]glyceraldehyde 3-phosphate for tritiation in position 4 or from [3-^{3}H]glyceraldehyde 3-phosphate for tritiation in position 5; and subsequently the tritiated 2C-methyl-D-erythritol 4-phosphates may be obtained enzymatically from the corresponding tritiated 1-deoxy-D-xylulose 5-phosphates.

Specifically, [1-^{3}H] glyceraldehyde 3-phosphate can be synthesized from [3-^{3}H] glucose by enzymatic action of hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase and triose phosphate isomerase. The reaction mixture containing [1-^{3}H]glyceraldehyde 3-phosphate can be directly used for synthesis of [3-^{3}H]1-deoxy-D-xylulose 5-phosphate. [3-^{3}H]1-deoxyDxylulose 5-phopshate can be synthesized from [1-^{3}H]glyceraldehyde 3-phosphate and pyrurate by enzymatic action of 1-deoxy-D-xylulose 5-phosphate synthase.

[1-^{3}H]2C-methyl-D-erythritol 4-phosphate can be synthesized from [3-^{3}H]1-deoxy-D-xylulose 5-phosphate by catalytic action of 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

[2-^{3}H]glyceraldehyde 3-phosphate can be synthesized from [2-^{3}H]glucose by enzymatic action of hexokinase, phosphoglucose isomerase, phosphofructokinase and aldolase. The reaction mixture containing [2-^{3}H]glyceraldehyde 3-phosphate can be directly used for synthesis of [4-^{3}H]1-deoxy-D-xylulose 5-phosphate as described above for [3-^{3}H]1-deoxy-D-xyluose 5-phosphate. [3-^{3}H]2C-methyl-D-erythritol 4-phosphate can be synthesized from [4-^{3}H]1-deoxy-D-xylulose 5-phosphate by catalytic action of 1-deoxy-D-xylulose 5-phosphate

The substrate is preferably a labelled 1-deoxy-D-xylulose or its 5-phosphate or 5-pyrophosphate or a labelled 2C-methyl-D-erythritol or its 4-phosphate or 4-pyrophosphate.

The labelled substrate may be labelled by 14-carbon, 13-carbon, deuterium or tritium. These four labelling types may be used alone or in any combination such as a combination of 13-carbon and deuterium.

The labelled precursors may be non-radioactive or radioactive. In case of non-radioactive precursors ^{13}C-labelled or ^{2}H-labelled precursors are preferred. In case of radioactive precursors ^{14}C-labelled or ^{3}H-labelled precursors are preferred. They are added as aqueous solution. The concentration of the precursors in the aqueous solution may be 1 μM to 1 mM. The concentration of plastids corresponds to a total protein content of 0.1 mg/ml to 10 mg/ml preferably 0.2 to 5 mg/ml. In case of ^{14}C-labelled precursors the radioactivity is preferably at least 0.1 μ Ci and in case of ^{3}H-labelled precursors preferably at least 1 μ Ci.

The labelling with 14-carbon or 13-carbon may be single whereby any one of the C-positions may be labelled. Alternatively, the substrates may be multiply labelled, such as dual, triple, quadruple or quintuple. The total C-labelling is particularly preferred in case of 13-carbon labelling.

The labelling with deuterium or tritium may be single or multiple. 1-deoxy-D-xylulose 5-phosphate may be deuterium- or tritium-labelled in positions 1, 3, 4 or 5, preferably in positions 3, 4 or 5; and 2C-methyl-D-erythritol 4-phosphate may be deuterium- or tritium-labelled in positions 1, 3, 4 or the methyl group.

Other intermediates downstream from 2C-methyl-D-erythritol 4-phosphate with corresponding labelling may be used.

reductoisomerase.

Deuterium-labelled, ^{13}C-labelled or ^{14}C-labelled substrates may be prepared analogously.

The basic enzymatic processes are known from G.A. Sprenger et al., Proc.Natl.Acad.Sci USA 94, 12857-12862 (1997); and Kuzuyama et al., Tetrahedron Lett. 39, 44509-4512 (1998).

Alternatively, the labelled 1-deoxy-D-xylulose compounds may be prepared by using the correspondingly labelled starting materials in the process described by Yokota, A and Sasajima, K. in Agric. Biol. Chem. 48, 149-158(1984) and ibid. 50, 2517-2524 (1986).

The labelled 2C-methyl-D-erythritol compounds may be obtained chemically by the following process, using correspondingly labelled starting materials:

(a) Reaction of 1,2 : 5,6-Di-O-ispropylidene-D-mannitol with lead tetracetate to isopropylidene glyceraldehyde; which is

(b) subsequently converted to 1,2-O-isopropylidene-(2R,3RS)-1,2,3-butanetriol by reaction with methyl magnesium iodide;

(c) Formation of 3,4-O-isopropylidene-(3R)-3,4-dihydroxy-2-butanone from the product step (b) by oxidation, preferably with sodium periodate in the presence of ruthenium dioxide;

(d) Formation of 1,2-O-isopropylidene-3-O-trimethylsilyl-(2R,3RS)-1,2,3-trihydroxy-3-cyano-butane by reacting the product of step (c) with trimethylsilyl cyanide;

(e) Conversion of the product of step (d) to a mixture of 2C-methyl-D-erythrono-1,4-lactone and 2C-methyl-D-threono-1,4-lactone by hydrolysis with an acid;

(f) production of 2,3-O-isopropylidene-2C-methyl-D-erythrono-1,4-lactone by reaction of the products of step (e) with acetone in the presence of anhydrous zink chloride;

(g) Conversion of the product of step (f) to 2,3-O-ispropylidene-2C-methyl-D-erthrofuranose by reaction with a hydride donor, preferably diisobutylaluminum hydride;

(h) Conversion of the product of step (g) to 2,3-O-isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime by reaction with O-benzylhydroxylamine;

(i) reaction of the product of step (h) with tribenzylphosphite and iodine to obtain 2,3-O-isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime 4-dibenzylphosphate;

(j) Conversion of the product of step (i) to 2,3-O-isopropylidene-2C-methyl-D-erytrhose 4-dibenzylphosphate by ozonization;

(k) Conversion of the product of step (j) to 2,3-O-isopropylidene-2C-methyl-D-erythritol 4-dibenzylphosphate by reaction with sodium borohydride;

(l) Converting the product of step (k) into 2C-methyl-D-erythritol 4-phosphoric acid by hydrogenation, preferably in the presence of palladium.

Tritiation in position 1 is possible by carrying out step (k) with tritiated sodium borohydride under otherwise identical conditions for the subsequent step (l). Tritiation in position 2' is possible by carrying out step (b) with tritiated methyl magnesium iodide prepared from tritiated methyl iodide and magnesium. The subsequent steps (c) to (l) remain unchanged. The combination of the tritiation steps is possible affording 2C-methyl-D-erythritol 4-phosphate acid tritiated in positions 1 and/or 2'.

Deuterium labelled, ^{13}C-labelled or ^{14}C-labelled substrates may be prepared analogously.

Total C-labelling can be carried out advatangeously starting from [U-^{13}C] glucose and [U-^{13}C] sodium pyruvate or [2,3-^{13}C]pyruvate. In the presence of thiamine pyrophosphate, ATP and $MgCl_2$ the following enzymes are used for preparing [U-^{13}C]1-deoxy-D-xylulose 5-phosphate; triose phosphate isomerase, hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, 1-deoxy-D-xylulose 5-phosphate synthase. Subsequently, the product can be converted to [U-^{13}C] 2C-methyl-D-erythritol 4-phosphate with 1-deoxy-D-xylulose 5-phosphate reductoisomerase and glucose, $NADP^+$ and $MgCl_2$.

The terpenoid separation in the screening method may be preferably carried out by disintegration of the cells or plastids and extraction of the lipophilic products, with or without the solid components in the mixture to be extracted.

The separation of the terpenoid fraction may be done by extraction with a lipophilic organic solvent, preferably chloroform, dichloromethane, ethylacetate. The extract is adjusted to a desired volume of e.g. 300 μl. An aliquot, e.g. 10 μl is used for liquid scintillation counting. The same is done with the mixture before incubation. The value before incubation is set to 100% and the values after incubation are given as % relative to the value before incubation. It is possible to separate from the extract individual terpenoids, such as carotenes by HPLC, thin layer chromatography or the like. In case of radioactive labelling, the determination of labelled product may be effected by a scintillation counter, by a phosphorimager, by a radio thin layer counter or by a radio detector in combination with a chromatographic column. In case of non-radioactive labelling, the determination may be effected conventionally by NMR spectroscopy (e.g. ^{13}C-NMR) or mass spectroscopy (e.g. HPLC-MS or GC-MS).

We have surprisingly found that in case of a use of tritiated starting materials, e.g. [1-^{3}H]2C-methyl-D-erythritol 4-phosphate, about 15% of the tritium label is

released into the ambient water. We further found that inhibitors of the deoxyxylulose phosphate pathway impair this tritium release. Therefore, by measuring the tritium release with and without a propsective inhibitor a rapid screening is possible. This rapid screening may be used as a pre-screening method. Subsequently, it can be tested whether a found inhibitor inhibits the pathway between 2C-methyl-D-erythritol 4-phopshate and isopentenyl pyrophosphate (IPP) or between isopentenyl pyrophosphate and phytoene. It has been ascertained that the addition of a phytoene desaturase inhibitor to the assay mixture does not affect the tritium release.

The new rapid screening method is very simple. It can be performed with a large number of test compounds, serially or in parallel. The method can be readily adapted to robotics.

It is required that the starting material contains at least one releasable tritium atom bonded to a carbon atom. Preferably, the tritium should be releasable only enzymatically but not chemically under the incubation conditions, and notably releasable by at least one enzyme in the terpenoid pathway. Perferably, the tritium should be stoichiometrically releasable by the reaction with an enzyme operating in the alternative pathway between 1-deoxy-D-xylulose 5-phosphate and IPP.

The 1-deoxy-D-xylulose or its 5-phosphate or 5-pyrophosphate is preferably tritiated in position 3 and/or in position 4; or less preferred in position 1. The 2C-methyl-D-erythritol or its 4-phosphate or 4-pyrophospate is preferably tritiated in position 1 and/or in position 3; or less preferred in the 2-methyl group. Other intermediates downstream from 2C-methyl-D-erythritol 4-phosphate with corresponding labeling may be used.

The overall enzymatic reaction sequence with tritium release by using any such

substrate is shown in Figure 3.

A laboratory experimental setup is shown in Figure 4. It comprises a glass scintillation vial 2 with a rubber stopper 4. A disc of filter paper 6 is attached with a pin 8 to the bottom of the stopper 4. The disc of filter paper has a first, larger zone 10 impregnated with an aliquot of the reaction mixture and a second, smaller zone 12, impregnated with a solution of a moisture indicator, such as a cobalt salt, notably $CoCl_2$. The vial 2 is placed upright into a Dewar tray or vessel 14 filled with a layer of a coolant such as liquid nitrogen, or solid carbon dioxide. This causes the water content in both zones 10 and 12 to be transported to the cold bottom of the vial 2, where it condenses to ice crystals 16. The transport direction is indicated by arrows 18.

Any other absorbing body may be used instead of the disc of filter paper and any tightly closing vessel may be used instead of the vial. The vial and the tray may be combined into a unitary structure. The bottom of the vial may contact the coolant directly or via a heat transmission body of, say metal.

For accelerating the sublimation of the water, the paper disc 6 may be irradiated with an infrared lamp. The duration of sublimation and/or irradiation may be determined by the change of color of the indicator zone 12, in case of Co^{2+} from red to blue. Subsequently, this vial is removed from the tray and opened and scintillation fluid is added. Any conventional scintillation fluid can be used. Preferably, Rotiszint 11® (consisting of toluene, 2,5-Diphenyloxazol and 1,4-bis[2-(5-phenyloxazolyl)]-benzene) is used. For determining the radioactivity a scintillation counter is used.

The arrangement may also be reversed, whereby the water vapor flows from the bottom of a vessel to the cooled top; or the vapor may flow horizontally.

For simultaneously carrying out a large number of tests (Fig.5) a plate 2 with a multitude of wells 4 may be used for charging the components 6 for the plastid reaction (Fig.5a). Subsequently, a similar plate with a corresponding array of wells 10 may be superimposed upside down on the first plate 2 in a sealed manner, so that dual wells are formed and isolated from each other. For this purpose, the surfaces of the plates 2,8 may be highly planar. Alternatively, a sealing gasket 12, for example, a correspondingly perforated plastic sheet may be interposed (Fig.5b).

Subsequently, the plastid reaction is carried out during a predetermined period of time, preferably, while the lower plate 2 is heated as indicated by heat plate 14.

After a predetermined incubation time, the upper plate 8 is cooled by a coolant such as dry ice or liquid nitrogen. For this purpose, the upper side of the upper plate may have the shape of a tray or a heat transmission plate 16 of, say metal, may be used. Now water vapor is condensed in the upper wells 10 as indicated by arrows 18 to form ice crystals 20. After a predetermined period of time, the plates 2 and 8 are separated and the upper plate 8 is inverted (Fig.5d) and scintillation fluid 22 is charged. Finally, the radioactivity in the wells is measured by scintillation measurement as symbolized in Figure 5e. A subset of the wells 4 may be charged with a small volume of a solution of a Co^{2+}-salt, such as $CoCl_2$. This may be used as an indicator for setting the evaporation time and amount.

Incubation is done preferably at 20 to 40°C and especially at 25 to 30°C. The incubation time may be 30min to 15h, preferably 2 to 10h, especially 6 to 8h.

For additionally determining the terpenoid formation, separate incubations may be used or a single incubation may be subdivided. The separation of the terpenoid fraction may be done by extraction with a lipophilic organic solvent, preferably chloroform, dichloromethane, ethylacetate. The extract is adjusted to a desired

volume of e.g. 300 µl. An aliquot, e.g. 10 µl is used for liquid scintillation counting. The same is done with the mixture before incubation. The value before incubation is set to 100% and the values after incubation are given as % relative to the value before incubation.

The chemical test compound may be added in substance or as a solution. In case of a water-soluble compound an aqueous solution is preferred. In other cases solution in hydrophilic protonic organic solvents, such as methanol, ethanol, propanol or hydrophilic aprotic organic solvents, such as dimethyl sulfoxide, dimethyl formamide, tetrahydrofuran, dioxane, ethylene glycol dimethyl ether, diethylene glycol dimethyl ether, triethylene glycol dimethyl ether, tetraethylene glycol dimethyl ether, hexamethylphosphoric triamide may be used. As solution mediators such solvents may also be added to a medium for the suspension of the plastids.

The remaining water phase can be analysed by HPLC, e.g. reversed phase ion-pair radio-HPLC (Anal. Biochem. 215, 142-149 (1993)) for detecting hydrophilic intermediates.

Preferably, the methods of the present invention are adapted to a high-throughput screen, allowing a multiplicity of compounds to be tested in a single assay. Such inhibitory compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall,UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant

and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondell et al., TibTech 14:60, 1996). Inhibitor assays according to the present invention are advantageous in accommodating many different types of solvents and thus allowing the testing of compounds from many sources.

Once a compound has been identified by the methods of the present invention as inhibitor, *in vivo* and *in vitro* tests may be performed to further characterize the nature and mechanism of the inhibitory activity. For example, the effect of an identified compound on *in vitro* enzymatic activity of purified or partially purified enzyme may be determined and enzyme kinetic plots may be used to distinguish, e.g., competitive and non-competitive inhibitors.

Compounds identified as inhibitors using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in commercial herbicide applications, etc. These modifications are achieved and tested using methods well-known in the art.

Any compound operating as inhibitor in this screening method is a herbicide. It can be used for specifically blocking the pathway to terpenoids via 1-deoxy-D-xylulose 5-phosphate in algae or plants.

The invention will now be explained with reference to examples.

Preparation Examples

Preparation Example 1

(a) 1,2-O-Isopropylidene-(2R,3RS)-1,2,3-butanetriol (7).

1,2:5,6-Di-O-isopropylidene-D-mannitol (**5**) (14 g, 53.4 mmol) was dissolved in 200 ml of dry chloroform. Anhydrous potassium carbonate (50.5 g, 366 mmol) was added, and the suspension was cooled to 0 °C. Lead tetraacetate (27.1 g, 61.1 mmol) was added in small portions under vigorous stirring. The orange coloured suspension was allowed to stand at room temperature over night. Potassium carbonate was filtered off by suction, and the filter cake was washed repeatedly with ether. The combined filtrate and washings were dried with magnesium sulphate and the solvent was removed under reduced pressure. The oil containing the isopropylidene glyceraldehyde was distilled quickly (60 °C at 30-40 mbar) affording 10,5 g (80,7 mmol, 76 %) of pure isopropylidene glyceraldehyde (**6**). The product was immediately dissolved in 35 ml of dry ether to avoid polymerisation. The solution of isopropylidene glyceraldehyde was added to a cooled solution of methyl magnesium iodide prepared from 5,1 g (207 mmol) of magnesium and 13,0 ml (209 mmol) of methyl iodide in 140 ml ether. After the aldehyde was added completely, the solution was stirred at room temperature over night. The solution was then slowly poured on crushed ice, and precipitated magnesium hydroxide was dissolved by the addition of saturated ammonium chloride (50 ml). The organic layer was removed, and the water phase was saturated with sodium chloride and extracted with chloroform (3 x 50 ml). The combined organic layers were dried with magnesium sulphate, and the solvent was removed under reduced pressure affording 9,9 g (67,8 mmol, 84 %) of 1,2-O-Isopropylidene-(2R, 3RS)-1,2,3-butanetriol (**7**).

^{1}H NMR (360 MHZ, $CDCl_3$): δ (ppm) 0.96 (d, ^3J = 6.5 Hz), 1,07 (d, ^3J = 6.5 Hz),

1.24 (s), 1.25 (2), 1.29 (s), 1.33 (s), 3.41-3.47 (m), 3.67-3.78 (m), 3.82-3.97 (m), 4.67 (d, ^3J = 4.6 Hz), 4.75 (d, ^3J = 5.2 Hz) (underlined signals belong to the diastereomer which is formed predominantly); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 18.0, 19.8, 24.8, 26.1, 64.3, 65.9, 66.1, 66.9, 79.1, 79.3, 107.7, 107.7 (underlined signals belong to the diastereomer which is formed predominantly; anal. calcd. for $C_7H_{14}O_3$: C 57.5, H 9.9, O 32.6 found: C 57.2, H 9.9, O 32.8.

(b)3,4-O-Isopropylidene-(3R)-3,4-dihydroxy-2-butanone (8)

1,2-O-Isopropylidene-(2R,3RS)1,2,3-butanetriol (**7**) (9.9 g, 67.8 mmol) was dissolved in 100 ml of chloroform. Water (100 ml), 30 g of potassium carbonate (217 mmol) and 50 mg of ruthenium dioxide hydrate were added. The suspension was stirred vigorously at room temperature, and 29 g (136 mmol) of sodium periodate were added in small portions. When the pH dropped below 7 it was adjusted to pH 8-8.5 with potassium carbonate. After the addition of periodate was complete the suspension was stirred for two days at room temperature. Before work up an aliquot of the reaction mixture was controlled by ^1H NMR spectroscopy. If starting material was still present, an additional amount of periodate was added. When the oxidation was complete the suspension was filtered by suction, and the filtrate was extracted with chloroform (4 x 50 ml). The combined organic layers were dried with magnesium sulphate, and the solvent was removed under reduced pressure affording 7.2 g (50 mmol, 74 %) of 3,4-O-Isopropylidene-(3R)-3,4-dihydroxy-2- butanone (**8**).

^1H NMR (250 MHz, $CDCl_3$): δ (ppm) 1.4 (s, 3H), 1.5 (s, 3H), 2.27 (s, 3H), 4.0 (dd, ^2J = 8.54Hz, ^3J = 5.50Hz, 1H), 4.2 (dd, ^2J = 8.54Hz, ^3J = 7.95Hz, 1H), 4.41 (dd, ^3J = 7.94Hz, ^3J = 5.5 Hz, 1H); ^{13}C NMR (62 MHz, $CDCl_3$): δ (ppm) 25.3 (CH_3), 25.6 (CH_3), 26.3 (CH_3), 66.4 (CH_2), 80.4 (CH), 110.9 (C_q).

(c) 1,2-O-Isopropylidene-3-O-trimethylsilyl-(2R,3RS)-1,2,3-trihydroxy-3-cyano-butane (9)

3,4-O-Isopropylidene-(3R)-3,4-dihydroxy-2-butanone (**8**) (7.2 g, 50 mmol) was dissolved in 50 ml of dry dichloromethane. Catalytic amounts of potassium cyanide (20 mg) and 18-crown-6 (20 mg) were added. Under cooling with ice, 9.4 ml (70 mmol) of trimethylsilyl cyanide were added within 20 minutes. The solvent and excess trimethylsilyl cyanide were removed under reduced pressure. The orange coloured oily residue (12.0 g, 49.3 mmol, 99 %) was a mixture of the erythro and threo form of 1,2-O-isopropylidene-3-O-trimethylsilyl-(2R,3RS)-1,2,3-trihydroxy-3-cyano-butane (**9**) in a ratio of 3: 1 which did not contain significant amounts of other products.

^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 0.17 and 0.18 (2s,9H), 1.12 (s), 1.29 (s), 1.40 (s), 1.43 (s), 1.46 (s), 1.57 (s) (9H) 3.85-3.90 (m, 1H), 3.97-4.10,(m, 2H); ^{13}C NMR (90 MHz, $CDCl_3$): *erythro* δ (ppm) 1.2 (TMS), 24.0 (CH_3), 25.0 and 26.0 (($CH_3)_2$), 65.0 (CH_2), 80.4 (CH), 110.9 (CN); *threo* δ (ppm) -3.1 (TMS), 25.2 (CH_3), 26.2 and 26.4 (($CH_3)_2$), 66.4 (CH_2), 80.8 (CH), 120.7 (CN).

(d) 2C-Methyl-D-erythrono-1,4-lactone (11) and 2C-Methyl-D-threono-1,4-lactone (12).

1,2-O-Isopropylidene-3-O-trimethylsilyl-(2R,3RS)-1,2,3-trihydroxy-3-cyano- butane (**9**) (12.0 g, 49.3 mmol) was suspended in 30 ml of 25 % hydrochloric acid. Ethanol (10 ml) was added to improve the solubility of the lipophilic cyanohydrin. The reaction mixture was stirred at 45°C for 30 minutes and subsequently under reflux for three hours. The mixture became brown, and a precipitate of ammonium chloride was formed. The acid was neutralized with concentrated ammonia. The mixture was evaporated to dryness. The product mass was triturated with 50 ml

of methanol. Insoluble ammonium chloride was filtered off. Methanol was removed under reduced pressure. The residual oil contained the lactones **11** and **12**, and the open chain carboxylic acids (**10**). Lactonisation was brought to completion by boiling the residue with 50 % formic acid (30 ml) for 2 hours. When no more open chain carboxylic acids were present the reaction mixture was concentrated under reduced pressure. The residual oil was dissolved in a mixture of ethyl acetate, 2-propanol and water (5 ml, 65:23.5:11.5, v/v). The solution was placed on a column of silica gel (acidic form) and was developed with the ethyl acetate/2-propanol water mixture. Fractions were combined, and concentrated under reduced pressure. The residue was lyophilised. The residual colourless oil (5.9 g, 44,7 mmol, 91 %) contained 2C-methyl-D-erythrono-1,4-lactone and 2C-Methyl-D-threono-1,4-lactone in a ratio of about 3: 1 as determined by NMR spectroscopy.

2C-Methyl-D-threono-1,4-lactone ^{1}H NMR (250 MHz, CD_3OD): δ (ppm) 1.30 (s, 3H), 3.92 (dd, ^2J = 4.27 Hz, ^3J = 9.16 Hz, 1H), 4.13 (dd, ^2J = 4.27 Hz, ^3J = 5.50 Hz, 1H), 4.44 (dd, ^2J = 5.50 Hz, ^3J= 9.15 Hz 1H); ^{13}C NMR (63 MHz, CD_3OD): δ (ppm) 17.9 (CH_3), 73.1 (CH_2), 78.8 (CH), 85.8 (C_q), 161.8 (C_q); IR (film): 1770 cm^{-1}; anal. calcd. for $C_5H_8O_4$: C 45.4, H 6.0, 48.3 found: C 46.2, H 6.5, O 47.3.

2C-*methyl-D-erythrono-1,4-lactone* ^{1}H NMR (250 MHz, CD_3OD): δ (ppm) 1.33 (s, 3H), 4.00 (dd, ^2J = 1.83 Hz, ^3J = 4.27 Hz, 1H), 4.09 (dd, ^2J = 1.83 Hz, ^3J = 9.77 Hz, 1H), 4.38 (dd, ^2J = 4.27 Hz, ^3J= 10.38 Hz, 1H); ^{13}C NMR (63 MHz, CD_3OD): δ (ppm) 21.9 (CH_3), 73.6 (CH_2), 75.0 (CH), 75.8 (C_q), 164.9 (C_q); IR (film): 1770 cm^{-1}.

open chain carboxylic acids (isomeric mixture 1:1) ^{1}H NMR (250 MHz, D_2O): δ (ppm) 1.14 (s, 3H), 1,17 (s, 3H), 3,45 - 3,85 (m, 6H); ^{13}C NMR (63 MHz, D_2O):

δ (ppm) 19.8 (CH_3), 20.7 (CH_3), 64.9 (CH_2), 65.2 (CH_2), 70.1 (CH), 70.3 (CH), 77.8 (C_q), 77.9 (C_q), 182.5 (C_q), 182.8 (C_q).

(e) 2,3-O-Isopropylidene-2C-methyl-D-erythrono-1,4-lactone (13)

Anhydrous zinc chloride (14.1 g, 103 mmol) was dissolved in 100 ml of acetone. The solution was cooled with ice, and 5.9 g of a mixture of 2C-methyl-D-erythrono-1,4-lactone (**11**) (33.5 mmol) and 2C-methyl-D-threono-1,4-lactone (**12**) (11.2 mmol) dissolved in 13 ml acetone was added. After 18 hours the solution was diluted with 150 ml of chloroform. Zinc chloride and unreacted 2C-methyl-D-threono-1,4-lactone were removed by washing with water (3 x 100 ml). The organic layer was dried with magnesium sulphate, and the solvent was removed under reduced pressure affording pure 2,3-O-isopropylidene-2C-methyl-D-erythrono-1,4-lactone (**13**) (4.4 g, 25.6 mmol, 76 % from 2C-methyl-D-erythrono-1,4-lactone) as a colorless oil which crystallized at -20°C.

^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 1.33 (s, 3H), 1.37 (s, 3H), 1.48 (s, 3H), 4.24 (dd, ^3J = 3.54 Hz, ^2J = 11.06 Hz, 1H), 4.34 (dd, ^2J = 11.06 Hz, ^3J = 0 Hz, 1H), 4.41 (dd, ^2J = 3.50 Hz, ^3J = 0 Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 18.4 (CH_3), 26.5 (CH_3), 26.9 (CH_3), 68.9 (CH_2), 80.3 (CH), 81.4 (C_q), 113.0 (C_q), 176.7 (C_q).

(f) 2,3-O-Isopropylidene-2C-methyl-D-erythrofuranose (14).

2,3-O-Isopropylidene-2Cmethyl-D-erythrono-1,4-lactone (**13**) (2.2 g, 12.9 mmol) was dissolved in 60 ml of dry tetrahydrofurane. The mixture was cooled to -78°C under an atmosphere of nitrogen. A solution of di-isobutylaluminum hydride (1 M in hexane, 17 ml, 17 mmol) was added slowly. The solution was allowed to stand

in the cooling bath over night. Wet ether (180 ml) and wet silica gel (30 g) were added. The mixture was stirred for one hour and was allowed to warm to room temperature. The mixture was then filtered. The solution was dried with magnesium sulphate, and the solvent was removed under reduced pressure. The residual oil was purified by chromatography on silica gel with a mixture of hexane/ethyl acetate (1 :2, v/v) affording 2.0 g (11.5 mmol, 89 %) of 2,3-O-isopropylidene-2C-methyl-D-erythrofuranose (**14**) as an anomeric mixture (α:β = 1:1).

^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 1.29 (s), 1.30 (s), 1.34 (s), 1 35 (s), 1.37 (s) (18 H), 3.46 (dd, ^3J = 3.54 Hz, ^2J = 11.06 Hz, 1H), 3.55 (m, 1H), 3.78 (d, ^2J = 11.50 Hz, 2H), 3.84 (d, ^2J = 11.06 Hz, 1H), 3.97 (dd, ^3J = 3.80 Hz, ^2J = 10.40 Hz, 1H), 4.29 (dd, ^3J = 3.10 Hz, ^3J = 8.85 Hz, 2H), 4.52 (d, ^2J = 11.06 Hz, 1H), 5.13 (d, ^3J = 2.65 Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 19.4 (CH_3), 21.4 (CH_3), 26.3 (CH_3), 26.9 (CH_3), 27.2 (CH_3), 28.0 (CH_3), 67.1 (CH_2), 71 5 (CH_2), 84.9 (CH), 86.0 (C_q), 86.1 (CH), 91.4 (C_q), 101.4 (C_q), 103.3 (C_q), 112.4 (CH), 112.9 (CH).

(g) 2,3-O-Isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime (15).

2,3-O-Isopropylidene-2C-methyl-D-erythrofuranose (**14**) (0.5 g, 2.87 mmol) was dissolved in 12 ml of dry dichloromethane. Dry pyridine (1 ml) and 0.88 g (5.5 mmol) of O-benzylhydroxylamine hydrochloride were added in one portion. The hydroxylamine dissolved within 20 minutes, and the reaction mixture became turbid after 40 minutes. The mixture was stirred for 15 hours at room temperature and was evaporated to dryness under reduced pressure. The residue was suspended in a mixture of chloroform/ethyl acetate (1:4, v:v, 1 ml). The solution was placed on a silica gel column (1 cm x 30 cm). The product was eluted with the solvent mixture. Fractions containing 2,3-O-isopropylidene-

2C-methyl-D-erythrose-(O-benzyl)oxime were combined and the solvent was removed under reduced pressure affording 0.53 g (1.9 mmol, 66 %) of 2,3-O-isopropylidene-2C-methyl-D-erythrose(O-benzyl)oxime as colourless oil.

^{1}H NMR (250 MHz, $CDCl_3$): δ (ppm) 1.26 (s, 3H), 1.31 (s, 3H), 1.33 (s, 3H), 3.42-3.56 (m, 2H), 3.86 (dd, ^3J = 4.89 Hz, ^3J = 6.72 Hz, 1H), 4.92 (s, 2H), 7.15-7.25 (m, 5H), 7.32 (s, 1H); ^{13}C NMR (63 MHz, CDC): δ (ppm) 22,8 (CH_3), 26.6 (CH_3), 27.9 (CH_3), 60.7 (CH_2), 76.0 (CH_2), 80.5 (CH), 84.3 (C_q), 109.4 (C_q), 127.9 (CH), 128.2 (CH), 128.3 (CH), 137.2 (C_q), 152.0 (CH).

(h) 2,3-O-Isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime 4-dibenzylphosphate (16).

Tribenzylphosphite (1.3 g, 3.7 mmol) was dissolved in 20 ml of dry dichloromethane. The solution was cooled to -20°C. Iodine (0.96 g, 3.8 mmol) was added in one portion. The mixture was protected from light and was allowed to come to room temperature when the violet color had disappeared. 2,3-O-Isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime (15) (0.53 g, 1.9 mmol) was dissolved in 20 ml of dichloromethane, and 2.5 ml pyridine (31.6 mmol) was added. The solution was cooled to -20°C and the solution of dibenzyl iodophosphate was added slowly. The reaction mixture was stirred for 2 hours at room temperature and was washed subsequently with sodium hydrogen sulphate (30 %, w/v, 2 x 10 ml), a solution of sodium hydrogen carbonate (5 %, w/v, 10 ml), and water (10 ml). The organic phase was dried with magnesium sulphate. The solution was evaporated to dryness. The residue was suspended in a mixture of hexane/ethyl acetate (3: 1, v:v, 2 ml). The mixture was placed on a silica gel column (1 cm x 20 cm) which was developed with hexane/ethyl acetate (3 : 1, v/v) until benzyl iodide was completely washed out. The product was then eluted with a mixture of chloroform/ethyl acetate (1:4, v/v). Fractions were combined.

The solvent was removed under reduced pressure affording 0.73 g (1.35 mmol, 71 %) of 2,3-O-isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime 4-dibenzylphosphate.

^{1}H NMR (250 MHz, $CDCl_3$): δ (ppm) 1.31 (s, 3H), 1.37 (s, 3H), 1.39 (s, 3H), 3.90-3.99 (m, 3H), 4.94 (s, 1H), 4.97-5.02 (m, 6H), 7.24-7.33 (m, 15H); ^{13}C NMR (63 MHz, $CDCl_3$): δ (ppm) 22.0 (CH_3), 26.6 (CH_3), 28.0 (CH_3), 65.3 (d, $^2J_{cp}$ = 5.5 Hz, CH_2), 69.1-69.5 (m, CH_2), 76.2 (CH_2), 80.2 (C_q), 82.5 (d, $^3J_{CP}$ = 7.9 Hz, CH), 109.7 (C_q), 127.9-128.5 (CH), 135.6 (d, $^3J_{CP}$ = 6.8 Hz, C_q), 137.9 (C_q), 150.3 (CH); ^{31}P NMR (101 MHz, $CDCl_3$): δ (ppm) -0.8 (s).

(i) 2,3-O-Isopropylidene-2C-methyl-D-erythrose 4-dibenzylphosphate (17).

2,3-O-Isopropylidene-2C-methyl-D-erythrose-(O-benzyl)oxime 4-dibenzylphosphate (**16**) (0.26 g, 0.43 mmol) was dissolved in 15 ml of dichloromethane containing 2 ml of pyridine. The solution was cooled to -78°C and was ozonized for 7 minutes with an ozone flow of about 3 g/min (0.44 mmol). Nitrogen was then bubbled through the dark blue reaction mixture. When the blue color had vanished, 2 ml of dimethylsulfide were added. The mixture was allowed to stand at -78°C for 1 hour and was then brought to room temperature. Solvent and pyridine was removed under reduced pressure, and the crude oil was purified by column chromatography (silica gel; chloroform/ethyl acetate 1/4, v/v) affording 0.17 g (0.39 mol, 81%) of pure aldehyde.

^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 1.24 (s, 3H), 1.36 (s, 3H), 1.46 (s, 3H), 3.93-4.02 (m, 2H), 4.05-4.13 (m, 1H), 4.92-5.00 (m, 4H), 7.23-7.30 (m, 10H), 9.51 (s, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 19.7 (CH_3), 26.5 (CH_3), 27.8 (CH_3), 64.3 (d, $^2J_{CP}$ = 6.0 Hz, CH_2), 69.5 (m, CH_2), 82.7 (d, $^3J_{CP}$ = 8.7 Hz, CH), 85.1 (C_q), 110.9 (C_q), 126.8 (d, $^4J_{CP}$ = 14.5 Hz, CH), 127.9 (CH), 128.6 (CH),

135.6 (d, $^3J_{CP}$ = 7.3 Hz, C_q), 202.0 (CH); ^{31}P NMR (101 MHz, $CDCl_3$): δ (ppm) -1.0 (s).

(j) 2,3-O-Isopropylidene-2C-methyl-D-erythritol 4-dibenzylphosphate (18).

2,3-O-Isopropylidene-2C-methyl-D-erythrose 4-dibenzylphosphate (**17**) (85 mg, 0.2 mmol) was dissolved in 3 ml of dry methanol, and the solution was cooled to 0°C. Sodium borohydride, 20 mg, (0.5 mmol) was added in one portion. Water (5 ml) was added to destroy the excess of borohydride, and the mixture was adjusted to pH 5 with concentrated acetic acid. The suspension was extracted 4 times with 10 ml of chloroform, and the organic solution was washed with 20 ml of 5 % sodium hydrogen carbonate. The organic phase was dried with magnesium sulphate, and the solvent was removed under reduced pressure affording 85.5 mg (0.2 mmol, 100 %) pure **18**.

^1H NMR (250 MHz, $CDCl_3$): δ (ppm) 1.20 (s, 3H), 1.30 (s, 3H), 1.36 (s, 3H), 1.89 (s, broad, 2H), 3.34 m, 2H), 3.95 (dd, J = 4.70 Hz, J = 7.10 Hz, 1H), 4.08-4.20 (m, 2H), 5.00 (dd, J = 1.83 Hz, J = 8.55 Hz, 4H), 7.29 (m, 10H); ^{13}C NMR (63 MHz, $CDCl_3$): δ (ppm) 22.1 (CH_3), 26.4 (CH_3), 28.1 (CH_3), 65.0 (CH_2), 65.2 (d, $^2J_{CP}$ = 5.45 Hz, CH_2), 69.4 (dd,$^2J_{Cp}$ = 2.72 Hz $^2J_{CP}$ = 5.45 Hz, CH_2), 81.1 (d,$^3J_{CP}$ = 8.18 Hz, CH), 81.7 (C_q), 108.5 (C_q), 126.9 (CH), 128.6 (CH), 135.6 (d,$^3J_{CP}$ = 6.80 Hz, C_q); ^{31}P NMR (101 MHz, $CDCl_3$):δ (ppm) 0.5 (s).

(k) 2C-Methyl-D-erythritol 4-phosphoric acid (4).

2,3-O-Isopropylidene-2C-methyl-D-erythritol 4-dibenzylphosphate (**18**) (85.5 mg, 196 μmol) was suspended in 8 ml of a mixture containing 4 ml of methanol and 4 ml of water. A catalytic amount of palladium on charcoal was added, and the suspension was hydrogenated for 20 hours at atmospheric pressure. The catalyst

was removed by filtrating through a 0.2 μm membrane filter. The acidic solution (pH 2) was heated to 70°C for 60 minutes. Methanol was removed under reduced pressure at 40°C, and the residue was lyophilized affording 35.3 mg (163 μmol, 83 %) of the crude phosphoric acid. The phosphoric acid was dissolved in 1ml of water. The solution was placed on a Nucleosil SB_{10} HPLC column and was eluted with 0.5 M formic acid at a flow rate of 1 ml/min. The effluent was monitored refractometrically. Fractions containing the product (retention volume 15 ml) were pooled and freeze-dried affording 18.0 mg of pure **4**.

^{1}H NMR (500 MHz, D_2O): δ (ppm) 1.04 (s, 3H), 3.37 (d, ^2J = 11.77 Hz, 1H), 3.50 (d, ^2J = 11.78 Hz, 1H), 3.64 (dd, ^3J = 2.60 Hz, ^3J = 8.10 Hz, 1H), 3.77 (ddd, $^3J_{HP}$ = 6.20 Hz, ^3J = 8.10 Hz, ^3J = 10.80 Hz, 1H), 4.01 (ddd, ^3J = 2.50 Hz, $^3J_{HP}$ = 6.00 Hz, ^3J = 10.80 Hz, 1H), ^{13}C NMR (125 MHz, D_2O): δ (ppm) 18.2 (C_3), 65.9 (d,$^2J_{PC}$ = 5.14 Hz, CH_2), 66.2 (CH_2), 73.1 (d, $^3J_{CP}$ = 7.58 Hz, CH), 73.8 (C_q); ^{31}P NMR (101 MHz, D_2O): δ (ppm) 3.7 (s).

Preparation Example 2

[1-$^2H_1$]-2C-Methyl-D-erythritol 4-phosphoric acid (4)

2,3-O-Isopropylidene-2C-methyl-D-erythrose 4-dibenzylphosphate (**17**), 85 mg, (0.2 mmol) was dissolved in 3 ml of dry methanol, and the solution was cooled to 0°C. [^{2}H]-$NaBH_4$ (20 mg, 0.5 mmol) was added in one portion. Water (5 ml) was added to destroy the excess of borohydride, and the mixture was adjusted to pH 5 with concentrated acetic acid. The suspension was extracted 4 times with 10 ml of chloroform, and the organic solution was washed with 20 ml of 5 % sodium hydrogen carbonate. The organic phase was dried with magnesium sulphate, and the solvent was removed under reduced pressure affording 85.5 mg (0.2 mmol, 100 %) pure [1-$^2H_1$]-2,3-O-Isopropylidene-2C-methyl-D-erythritol 4-dibenzylphosphate (**18**).

[1-$^2H_1$]-2,3-O-Isopropylidene-2C-methyl-D-erythritol 4-dibenzylphosphate (85.5 mg, 196 μmol) was then suspended in 8 ml of a mixture containing 4 ml of methanol and 4 ml of water. A catalytic amount of palladium on charcoal was added, and the suspension was hydrogenated for 20 hours at atmospheric pressure. The catalyst was removed by filtrating through a 0.2 pm membrane filter. The acidic solution (pH 2) was heated to 70°C for 60 minutes. Methanol was removed under reduced pressure at 40°C, and the residue was lyophilized affording 35.3 mg (163 μmol, 83%) of the crude phosphoric acid. The phosphoric acid was dissolved in 1 ml of water. The solution was placed on a Nucleosil SB_{10} HPLC column and was eluted with 0.5 M formic acid at a flow rate of 1 ml/min. The effluent was monitored refractometrically. Fractions containing the product (retention volume 15 ml) were pooled and freeze-dried affording pure [1-$^2H_1$]-**4**.

Preparation Example 3

[1-$^3H_1$]-2C-Methyl-D-erythritol 4-phosphoric acid (4)

[^{3}H]-$NaBH_4$ (8.5 μmol, 100 mCi, 11.8 Ci/mmol) was suspended in 500 μl of dry methanol. 170 μl of a solution containing 33.3 μmol of 2,3-O-Isopropylidene-2C-methyl-D-erythrose 4-dibenzylphosphate (**17**) in dry methanol was added in one portion to the borohydride suspension at room temperature. After 1 hour at room temperature 1 ml of water was added to destroy unreacted borohydride. The resulting suspension was extracted with chloroform (3 x 170 μl), the organic phases were combined and the solvent was removed under reduced pressure without drying.

The residue was dissolved in 50 % methanol (1 ml), a catalytic amount of Pd on charcoal was added and the mixture was hydrogenated for 12 hours (room temperature, 1 atm). The catalyst was removed by filtration. Acetic acid (100 %, 1 ml) was added and the mixture was heated to 60°C for 30 minutes.

Preparation Example 4

The repetition of preparation example 1 with [^{13}C]methyl iodide in step (a) affords the ^{13}C-labelled product (**4**).

Preparation Example 5

The repetition of preparation example 1 with [$^{2}H_3$]methyl iodide affords the deuterium labelled product (**4**).

Preparation Example 6

The repetition of preparation Example 1 with [^{3}H] methyl iodide affords the tritium labelled product (4).

Preparation Example 7

The repetition of preparation example 1 with potassium [^{14}C] cyanide in step (c) affords ^{14}C-labelled product (**4**).

Preparation Example 8

[1,2-$^{14}C_2$] 1-Deoxy-D-xylulose 5-phosphate (specific activity: 62,5 m Ci/mmol) was prepared biosynthetically by the method described in Sprenger et al Proc. Natl. Acad. Sci. USA 94 (1997) 12857 - 12862, using [U-^{14}C]pyruvate (specific activity: 150 m Ci/mmol) and D,L-glyceraldehyde 3-phosphate.

Preparation Example 9

[1-^{3}H] 1-Deoxy-D-xylulose 5-phosphate (specific activity: 5 mCi/mmol) was synthesized in accordance with Preparation Example 8 by using [3-^{3}H]pyruvate (specific activity: 72.3 Ci/mmol).

Preparation Example 10

[1,2-$^{14}C_2$] 1-Deoxy-D-xylulose (specific activity: 62.5 mCi/mmol) was prepared from [U-^{14}C] pyruvate with a specific radioactivity of 150 mCi/mmol and D-glyceraldehyde by using as catalyst the pyruvate dehydrogenase complex of *E-coli* DH5α. The yield was 80%. The method of Yokota, A. and Sasajima, K. Agric. Biol. Chem. 48 149-158 (1984) and ibid 50, 2517-2524 (1986) was used.

Preparation Example 11

Enzymatic preparation of [3-^{3}H]1-deoxy-D-xylulose 5-phosphate.

A reaction mixture containing 166 mg D- [3-^{3}H]glucose (50 μCi, NEN Life Science products NET331), 44 mg thiamine pyrophosphate, 1.02 g of ATP, 200 mg of sodium pyruvate , 6 mM $MgCl_2$, in 150 mM Tris hydrochloride, pH 8.0 is prepared. 410 units of triose phosphate isomerase, 360 U hexokinase, 50 U phosphoglucose isomerase, 20 U phosphofructokinase, 35 U aldolase and 2 U recombinant DXP synthase from *B.subtilis* are added to a final volume of 58 ml. The reaction mixture is incubated at 37°C overnight. During the reaction the pH is held at a constant value of 8.0 by the addition of 1 M NaOH (2 ml). The solution contained the desired product.

Preparation Example 12

Enzymatic synthesis of [1-^{3}H]2C-methyl-D-erythritol 4-phosphate.

To 60 ml of the solution of Preparation Example 10, 10 U DXP reductoisomerase, 120 U glucose dehydrogenase, 0.97 g glucose, 200 mM $MgCl_2$ and 0.3 mM $NADP^+$ are added. The pH is adjusted to 8.0 and the final volume of the solution is 72 ml. The reaction mixture is incubated at 37°C overnight.

[1-^{3}H]2C-methyl-D-erythritol 4-phosphate was purified by HPLC using a column of

Nucleosil 10SB (Macherey & Nagel), size: 4.6 x 250 mm). The column was eluted with 0.2 M formic acid containing 10 mM ammonium formate at a flow rate of 0.7 ml/min. The eluent was monitored by a radiomonitor (Berthold). [1-^{3}H]2C-methyl-D-erythritol 4-phosphate was eluted at 16 ml. Fractions containing [1-^{3}H]2C-methyl-D-erythritol 4-phosphate were collected and lyophylized.

Preparation Example 13

Enzymatic preparation of [4-^{3}H]1deoxy-D-xylulose 5-phosphate.

A reaction mixture containing 166 mg D- [2-^{3}H] glucose (50 μCi, NEN Life Science products, NET 238C) 44 mg thiamine pyrophosphate, 1.02 g of ATP, 200 mg of sodium pyruvate, 6 mM $MgCl_2$, in 150 mM Tris hydrochloride, pH 8.0 is prepared. 360 U hexokinase, 50 U phosphoglucose isomerase, 20 U phosphofructokinase, 35 U aldolase and 2 U recombinant DXP synthase from *B. subtilis* are added to a final volume of 58 ml. The reaction mixture is incubated at 37°C overnight. During the reaction the pH is hold at a constant value of 8.0 by the addition of 1 M NaOH (2 ml). The solution contained the desired product.

Preparation Example 14

Enzymatic synthesis of [4-^{3}H]2C-methyl-D-erythritol 4-phosphate.

To 60 ml of the solution of Preparation Example 12 10 U DXP reductoisomerase, 120 U glucose dehydrogenase, 0.97 g glucose, 200 mM $MgCl_2$ and 0.3 mM $NADP^+$ are added. The pH is adjusted to 8.0 and the final volume of the solution is 72 ml. The reaction mixture is incubated at 37°C overnight. [4-^{3}H]2C-methylerythritol 4-phosphate is purified by HPLC as described above for [1-^{3}H]2C-methylerythritol 4-phosphate.

Perparation Example 15

Enzymatic synthesis of [U-^{13}C]1-deoxy-D-xylulose 5-phosphate

A mixture containing 166 mg [U-^{13}C]glucose, 44 mg thiamine pyrophosphate, 1.02 g of ATP, 200 mg of [U-^{13}C]sodium pyruvate or [2,3^{13}C]pyruvate, 6 mM $MgCl_2$, in 150 mM Tris hydrochloride, pH 8.0 is prepared. 410 units of triose phosphate isomerase, 360 U hexokinase, 50 U phosphoglucose isomerase, 20 U phosphofructokinase, 35 U aldolase and 2 U recombinant 1-deoxy-D-xylulose 5-phosphate synthase from *B. subtilis* are added to a final volume of 58 ml. The reaction mixture is incubated at 37°C overnight. During the reaction the pH is held at a constant value of 8.0 by the addition of 1M NaOH (2 ml). ^{13}C-NMR-spectra are recorded for monitoring the conversion.

Preparation Example 16

Enzymatic synthesis of [U-^{13}C]2C-methyl-D-erythritol 4-phosphate

To 60 ml of the reaction mixture of Preparation Example 11 10 U 1-deoxy-D-xylulose 5-phosphate reductoisomerase, 120 U glucose dehydrogenase, 0.97 g glucose, 200 mM $MgCl_2$ and 0.3 mM $NADP^+$ are added. The pH is adjusted to 8.0 and the final volume of the solution is 72 ml. The reaction mixture is incubated at 37 °C overnight. ^{13}C-NMR-spectra of the product are recorded for monitoring the conversion.

Plastid isolation examples

Isolation example 1

Chromoplasts of *Capsicum annuum L.*

The pericarp of *Capsicum annuum* (500 g fresh weight) was freed of seeds and chopped and 500 ml isolation meidum 1 was added at 4°C. The isolation medium1 contained the following components: 50mM Hepes buffer, pH 8.0; (1 mM dithioerythritol (77.1 mg/500 ml), 1 mM ethylene diaminotetracetic acid disodium salt (186.1 mg/500 ml) and 0.4 mM sucrose (68.46 g/500 ml). The fruits were homogenized in a Waring blender (3x for 1 second) and subsequently filtered through nylon gauze (4 layers, 50 μm). Cell fragments were removed by centrifugation for 5 minutes at 150 g (Sorvall centrifuge; GSA rotor). The supernatant was centrifuged at 2200 g for 10 min. The obtained chromoplast pellet was subsequently washed once with siolation medium The crude chromolats obtained in this matter had a protein concentration of 10 to 15 mg/ml (Bradford, Anal. Biochem. 72 248-254 (1976). The isolation method was modified from B. Camara, Methods Enzymol. 214 352-365 (1993).

Isolation Example 2

Chromoplasts of *Narcissus pseudonarcissus L.*

The method of Liedvogel et al, Cytobiology 12 155-174 (1976) was used.
50 inner coronae (about 50 g fresh weight) of *Narcissus pseudonarcissus* were used. 250 ml cold isolation medium 2 was added. (67 mM potassium phosphate buffer, pH 7.5; 5 mM $MgCl_2$ (254.1 mg/250 ml), 0.2 % polyvinylpyrrolidone K 90 (0.5 g/250 ml), 0.74 M sucrose (63.3 g/250 ml)). The mixture was homogenized in a Waring Blender (3 x for 1 second). After filtration of the homogenate through nylon gauze (3 layers, 50 μm) and centrifugation at 1400 g for 5 min (Eppendorf

centrifuge) a chromoplast pellet was obtained by further centrifugation of the supernatent at 16500 g for 20 min. (Sorvall centrifuge, GSA rotor). The pellet was resuspended in a buffer of the following composition: 50 % (w/v) sucrose in 67 mM potassium phosphate buffer, pH 7.5, 5 mM $MgCl_2$ (1-2 ml) and transferred into a transparent centrifugation tube. Solution of 40 %, 30 % and 15 % (w/v) sucrose in the same buffer were prepared and 11 ml of each solution was placed as a discrete layer above the liquid in the transparent centrifugation tube. After centrifugation (Beckmann, SW 28 rotor) at 50 000 g (60 min) the chromoplasts floatated and formed two bands at the interphases of 40/30 % (w/v) and 30/15 % (w/v) sucrose. The chromoplast bands were removed carefully with a pipette from the sucrose gradient, and diluted with buffer to a sucrose end concentration of 15 % (w/v) so that the plastids could be pelletized by centrifugation at 16 500 g for 20 min. (Sorvall, SS 34 rotor). Subsequently they were resuspended in incubation buffer (100 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 2 mM dithioerythritol). The protein concentration was determined by the method of Bradford.

Isolation Example 3

Chloroplasts

Green fruits of *Capsicum annuum L* were used to isolate chloroplasts by the method of isolation example 1 with the exception that the pellet was obtained by centrifugation at 2200 g for 2 min. (Sorvall, GSA rotor). Similarly chloroplasts of *Avena sativa* (250 g primary leaves) were isolated by the above method. The plants were grown for 7 days on vermiculit grannules (exposure: 1400 lx, 25°C, 50-60 % rH). By the same method chloroplasts of *Lactuca capitata var. capitata L., Spinacia oleracea L., Secale cereale L., Brassica oleracea subsp. oleracea var. italica L.* (from the buds of the inflorescence) *Oryza sativa* and *Zea mays* were isolated.

Screening Examples

Screening Example 1

Step A Assay without inhibitors

The chromoplasts of *Capsicum annuum L.* were used. The incubation was done in an Eppendorf Cap at 30 °C during 15 h. The total volume of the enzyme composition was 500 µl. It was composed as follows: 100 mM Hepes buffer, pH 7.6, 2 mM $MnCl_2$ (1 µmol), 10 mM $MgCl_2$ (5 µmol), 2 mM NADPH (1 µmol), 0.1 µCi [1,2-$^{14}C_2$] 1-deoxy-D-xylulose 5-phosphate (1.6 nmol; 342.4 ng).

The enzymatic reaction was started by the addition of chromoplast suspension (about 2 mg protein). In control tests the corresponding amount of distilled water was added.

For demonstrating that the radiolabelled substrates entered into carotenoids and other lipophilic compounds the enzyme compositions were extracted twice in 1 ml chloroform and the combined chloroform phases were concentrated with a Speed-Vac (Bachhofer). The volume was brought to 300 µl and a 10 µl portion was used for the determination of the radioactivity in the organic phase by means of a scintillation counter (Automatic Counter LS6500; Beckmann).

For demonstrating the radioactivity in a certain carotenoid fraction the organic phase was separated by means of thin layer chromatography. The evaluation was done by means of a phosphorimager (Raytest) or a radio thin layer scanner (Automatic TLC Linear Analyzer, Berthold). To obtain values of radioactivity for the total terpenoids in the organic phases or for the β-carotene, the percentage of radioactive labelled precursor converted into terpenoids or β-carotene, respectively, is calculated. The result is 14 % for all terpenoids or 10 % for β-carotene.

Step B Assay with inhibitors

The incubation of Step A was repeated with the addition of increasing amounts of 3-(N-formyl-N-hydroxyamino)propyl phosphonic acid monosodium salt (F1) (prepared in accordance with Öhler, E. and Kanzler, S. Synthesis 539-543 (1995); Yazawa, H. and Goto, S., Tetrahedron Letters 26 (3703-3706) (1985) or 3-(N-hydroxyamino) propylphosphonic acid monosodium salt (F2) (prepared in accordance with Öhler, E. and Kanzler, S. Synthesis 539-543 (1995). The results are shown in Table 1. As base value the result obtained in step A is set in Table 1 as 100.

Table 1

Inhibitor concentration μM	Conversion into Terpenoids (%)	
	Inhibitor F1	Inhibitor F2
0	100	100
0.1	88	100
1	66	100
3	45	100
5	30	100
10	6	100
100	3	100
1000	0	100

The results show that the screening method is sensitive for the detection of inhibitors and for the discrimination of inhibitors from non-inhibitors.

Screening Example 2

Screening Example 1 is repeated with the exception that ^{14}C-labelled 1-deoxy-D-xylulose or ^{3}H-labelled 2C-methyl-D-erythritol 4-phosphate is used instead of ^{14}C-labelled 1-deoxy-D-xylulose 5-phosphate. The results without inhibitor are analogous, whereby the incorporation of 1-deoxy-D-xylulose is about 10 % of the incorporation of 1-deoxy-D-xylulose 5-phosphate. The results with inhibitor are the same as in Screening Example 1 for 1-deoxy-D-xylulose but no inhibition was found for 2C-methyl-D-erythritol 4-phosphate.

Screening Example 3

Screening Example 1 is repeated with the chromoplasts of Narcissus pseudonarcissus L. or with the chloroplasts of *Capsicum annuum L.*, *Avena sativa L.*, *Lactuca capitata var. capitata L.*, *Spinacia oleracea L.*, *Secale cereale L.*, *Brassica oleracea, supsp. oleracea var. italica L.*, *Oryza sativa*, *Zea mays*. Analogous results are obtained.

Screening Example 4

Screening Example 1 is repeated with [1-^{14}C] isopentenyl pyrophosphate (0.1 μCi, 1.8 nmol). It was found that neither F1 nor F2 showed inhibition.

Screening example 5

Step A without inhibitors

The chromplasts of *Capsicum annuum L.* were used. The incubation was done in an Eppendorf Cap at 30°C during 15h. The total volume of the reaction composition was 500 μl. It was composed as follows: 100 mM Hepes buffer, pH 7,6, 2 mM $MnCl_2$ (1μmol), 10 mM $MgCl_2$ (5 μmol), 2 mM NADP (1 μmol), 20 μM FAD (10 nMol), 5 mM NaF (2.5 μmol), 6 mM ATP (3 μmol), 1 mM NADPH (0.5 μmol), 5 μCi [1-^{3}H]2C-methyl-D-erythritol 4-phosphate (MEP) 6.66 nmol; 1.45 μg).

The enzymatic reaction was started by the addition of 150 µl chromoplast suspension (about 2 mg of protein). In control tests the same amount of distilled water was added.

To demonstrate that trititium is lost to the medium during the conversion of [1-^{3}H]2C-methyl-D-erythritol 4-phosphate into terpenoids the following procedure was carried out (method according to Mitra, R., Gross, R.D. and Varner, J.E. Anal. Biochem. 64, 102-109 (1975); Amrhein, N., Gödeke, K.-H. and Gerhardt, J. Planta, 131, 33-40 (1976); the method is shown in Fig.4): a 50 µl aliquot of the reaction mixture was spotted onto a disk of Whatman paper (⌀10 mm) and 5 µl of a 750 mM solution of $CoCl_2$ was spotted in a separate location. The disk of filter paper was fixed with a pin at the bottom of a stopper which was used to seal a glass scintillation vial tightly. This vial was placed into a Dewar vessel filled with liquid nitrogen so that only the bottom is cooled. The paper disk was heated with an infrared lamp. H_2O together with ^{3}HOH sublimated to the cooled bottom.

When the sublimation was completed (indicated through the change of the color of the $CoCl_2$-solution from red to blue) the vial was removed from the liquid nitrogen, the stopper was carefully removed from the vial and scintillation fluid was added. For the determination of the radioactivity a scintillation counter was used (Automatic Counter LS6500; Beckmann).

The value of radioactivity before incubation is set is set to 100% and the values obtained for the ^{3}HOH-release after incubation are given as % relative to the value before incubation. The results are given in Table 2.

To show that the radiolabelled substrates entered into terpenoids the rest of the enzyme assay was extracted twice with 1 ml of ethyl acetate and the combined ethyl acetate phases were concentrated with a Speed-Vac (Bachhofer). The

volume was brought to 300 µl and a 10 µl aliquot was used for the determination of the radioactivity in the organic phase by means of a scintillation counter.

To obtain values of radioactivity for the total terpenoids in the organic phase, the percentage of conversion of radioactive labelled percursors converted into terpenoids is calculated. The results are also given in Table 2.

Step B with inhibitors

The incubation of Step A was repeated with the addition of norflurazon or 2-(dimethylamino)ethyl diphosphate (DMAEPP) prepared in accordance with Muehlbacher M. and Poulter C.D., Biochem. 27, 7315-7328 (1988)). The results are shown in Table 2.

Screening Example 6

Screening example 5 is repeated with 0.1 µCi [2-^{14}C] 2C-methyl-D-erythritol 4-phosphate (9.6 nmol; 2.08 µg). The result is given in Table 2 (to make sure that there are no volatile compounds responsible for the radioactivity in the sublimated water).

Table 2

Enzyme assay	^{3}HOH-release (%)	Conversion into terpenoids (%)
5 μCi [1-^{3}H] MEP 4h	4	2
5 μCi [1-^{3}H] MEP 15h	14	10
5 μCi [1-^{3}H] MEP 15h + 100 μM Norflurazon	13	9
5 μCi [1-^{3}H] MEP 15h + 2 mM DMAEPP	1.4	1.9
0.1 μCi [2-^{14}C] MEP 4h	0.7	13
0.1 μCi [2-^{14}C] MEP 15h	0.7	35
5 μCi [1-^{3}H] MEP 15h, without chromplasts (control)	0.65	0

Screening Example 7

Run 7/1

The screening procedure of Example 1 was repeated with the exception of using 1 ml ethylacetate in place of 1 ml chloroform. Conversions into terpenoids as well as into β-carotene + phytoene were determined. The results are given in Table 3.

Runs 7/2 to 7/9

Run 1 was repeated with the addition of nucleotides as specified in Table 3. The conversions are also given in Table 3. Runs 7/2 and 7/5 in comparison to run 7/1 show that ATP and CTP are both promotors. Run 7/8 shows that ATP and CTP jointly are synergistic. Run 7/4 shows that CDP can be used as a source for CTP. It seems to be converted by plastidal enzymes to CTP by phosphorylation with ATP. Run 7/7 shows again the synergism compared to runs 7/1, 7/2, 7/4. Run 7/3 shows that by the addition of CMP alone the yield of terpenoids decreases as compared to run 7/1, which may be due to a depletion of ATP in the plastids by

conversion to mainly CDP and not CTP. Run 6/6 shows that a combination of ATP and CMP is also synergistic, probably since the amount of ATP is sufficient for the enzymatic conversion of CDP to CTP and as promotor. Run 7/9 shows that run 7/6 is close to saturation. A repetition of runs 7/1 to 7/9 with the inhibitors and conditions of screening example 1 produces inhibition results similar to those in Table 1.

Table 3

Run	Nucleotides (mM)				Conversion to terpenoids (%)	Conversion to β-carotene + phytoene (%)
	ATP	CMP	CDP	CTP		
7/1	-	-	-	-	3.7	0.74
7/2	6	-	-	-	22	10.2
7/3	-	6	-	-	1.2	0.03
7/4	-	-	6	-	8.5	2.6
7/5	-	-	-	6	14.5	6.5
7/6	3	3	-	-	43.8	25.6
7/7	3	-	3	-	45.3	25.7
7/8	3	-	-	3	48.7	28.3
7/9	6	-	-	6	49.8	34.9